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Key Divisions in the Early *Arabidopsis* Embryo Require POL and PLL1 Phosphatases to Establish the Root Stem Cell Organizer and Vascular Axis

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SUMMARY

***Arabidopsis* development proceeds from three stem cell populations located at the shoot, flower, and root meristems. The relationship between the highly related shoot and flower stem cells and the very divergent root stem cells has been unclear. We show that the related phosphatases POL and PLL1 are required for all three stem cell populations. *pol pll1* mutant embryos lack key asymmetric divisions that give rise to the root stem cell organizer and the central vascular axis. Instead, these cells divide in a superficially symmetric fashion in *pol pll1* embryos, leading to a loss of embryonic and postembryonic root stem cells and vascular specification. We present data that show that POL/PLL1 drive root stem cell specification by promoting expression of the *WUS* homolog *WOX5*. We propose that POL and PLL1 are required for the proper divisions of shoot, flower, and root stem cell organizers, *WUS/WOX5* gene expression, and stem cell maintenance.**

INTRODUCTION

Stem cell divisions are often asymmetric and produce a self-renewing stem cell and a progeny cell destined to undergo differentiation. Stem cells are usually maintained by a non-stem-cell microenvironment, or niche, that provides proper positional cues (Spradling et al., 2001). In plants, two stem cell niches are distally present in specialized structures, the shoot and root meristems. Both are established during the embryogenesis and function as ultimate sources for postembryonic growth (Laux, 2003; Weigel and Jurgens, 2002).

Embryogenesis of *Arabidopsis* is an ordered developmental process by which the basic body pattern, including the shoot and root stem cells, is established (Jürgens, 2001). The vast majority of plant development results from postembryonic organogenesis from the shoot and root stem cells. The continuous de novo organogenesis at the meristems allows plants to carry out developmental processes with a great amount of plasticity and flexibility.

Stem cell niches formed during embryogenesis have been identified. At the shoot meristem, a group of cells immediately underlying the stem cells express the transcription factor WUSCHEL (WUS), establishing the meristem organizing center (OC) (Laux, 2003; Mayer et al., 1998). The OC, in a fashion that is not well understood, maintains stem cells in the overlying cell layers (Schoof et al., 2000). The expression of *WUS* is negatively regulated by the CLAVATA (CLV) signaling pathway, composed of CLV1, CLV2, and CLV3 (Brand et al., 2000; Schoof et al., 2000). CLV1 is a receptor-like kinase, CLV2 is a receptor-like protein, and CLV3 is a putative polypeptide ligand (Clark et al., 1997; Fletcher et al., 1999; Jeong et al., 1999).

At the root meristem, a group of mitotically inactive cells termed the quiescent center (QC) acts in part as a niche for the surrounding root stem cells (Aida et al., 2004; Dolan et al., 1993; van den Berg et al., 1997). The QC requires two pathways for its formation and maintenance: the auxin-independent activity of the GRAS-type putative transcription factors SCARECROW (SCR) and SHORTROOT (SHR) intersecting with the auxin maximum established in the basal end of the embryo controlling the expression of *PLETHORA1* (*PLT1*) and *PLT2*, encoding AP2-type putative transcription factors (Aida et al., 2004; Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 1999).

Although there is no obvious morphological similarity between shoot and root meristems, both the OC and the QC function similarly in suppressing the differentiation of adjacent stem cells. However, the regulatory pathways characterized at these stem cell populations are almost entirely nonoverlapping, raising questions as to the mechanistic and evolutionary relationships between these two key plant structures. Two observations have suggested that there might exist a link between these two structures. First, ectopic expression of CLV3 and related CLE proteins induced early termination of both shoot and root meristems, suggesting that CLV1-related receptors might regulate both shoot and root meristem development (Casamitjana-Martinez et al., 2003; Fiers et al., 2005; Hobe et al., 2003; Ito et al., 2006). The *WUS* homolog *WOX5* was recently shown to play crucial roles in the proliferation of the stem cell population distal to the QC (Sarkar et al., 2007). Along with that of other regulators, *WOX5* activity is required for the maintenance of stem cells proximal to the QC (Sarkar et al., 2007).

The type 2C protein phosphatases POLTERGEIST (POL) and PLL1 play crucial roles in maintaining shoot stem cells both

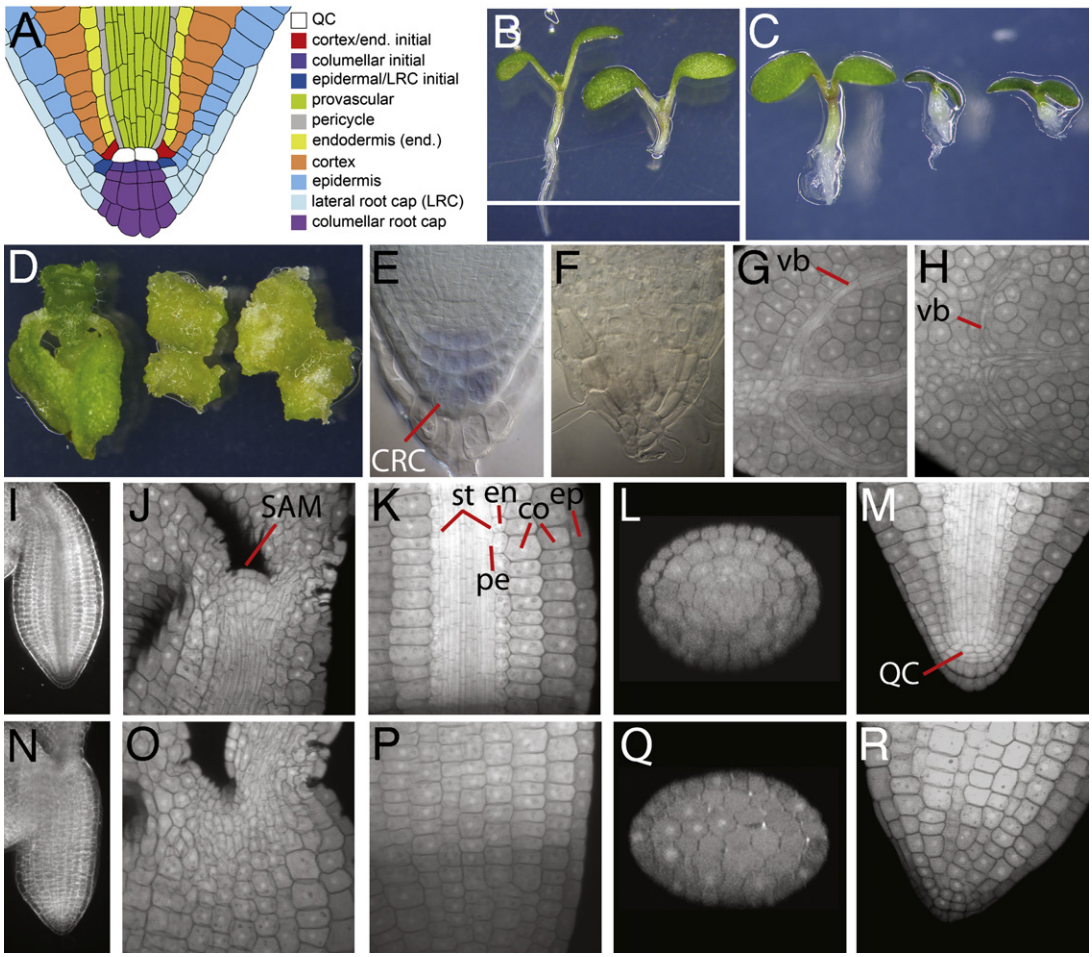

Figure 1. *pol pll1* Double Mutants Lack a Root Meristem and Vascular Axis

(A) An image of a wild-type embryonic root outlined and color coded for specific cell types within the meristem.

(B) Wild-type (left) and *pol pll1* (right) seedlings 5 days after germination in the light.

(C and D) Comparisons of auxin-resistant *axr1-13* (left), wild-type (center), and *pol pll1* (right) seedlings (C) grown for 5 days on the auxin mimic 2,4-D (900 pM) and (D) grown for 17 days on callus-inducing media.

(E and F) (E) Wild-type and (F) *pol pll1* root tips were stained with lugol solution to mark the columella root cap (CRC).

(G–R) Mature embryos dissected from seeds of *pol/pol pll1*/+ plants. (I and N) Dark-field images and (G, H, J–M, and O–R) confocal laser scanning microscopic (CLSM) images of (G and H) cotyledon tips, (J and O) shoot apices, (K and P) medial sections of hypocotyls, (L and Q) transverse sections reconstructed from longitudinal Z-series, and (M and R) medial sections of embryonic roots from (H and N–R) *pol pll1* mutants and (G and I–M) wild-type-like siblings. All wild-type plants were Columbia ecotype. SAM, shoot apical meristem; ep, epidermis; co, cortex; en, endodermis; pe, pericycle; st, stele; QC, quiescent center; vb, vascular bundle.

embryonically and postembryonically (Song and Clark, 2005; Song et al., 2006; Yu et al., 2003). Neither *pol* nor *pll1* single mutants have dramatic phenotypes; however, the *pol pll1* double mutants are lethal, leading to a lack of shoot and root growth in seedlings. The shoot growth defects are the result of the fact that POL and PLL1 are necessary for the maintenance of *WUS* expression and stem cell specification, and CLV1 signaling acts through POL/PLL1 to restrict *WUS* expression (Song et al., 2006). POL and PLL1 are the closest known factors to *WUS* transcriptional regulation in the shoot meristem.

In this report, we demonstrate that *pol pll1* double mutants lack embryonic and postembryonic specification of all stem cells, including those at the root meristem. The central vascular axis of the mutants is completely absent. These phenotypes were traced to the defects in the division and specification of procambial and hypophyseal cells early in embryogenesis. The lack of *pol pll1* root stem cells is in part the consequence of the loss of *WOX5* expression at the QC, suggesting a common mechanism for both root and shoot stem cell specification.

RESULTS

pol pll1 Mutants Lack All Embryonic Stem Cells

pol pll1 double mutants fail to form a functional embryonic shoot meristem and are seedling lethal, with defects in basal embryo patterning (Song et al., 2006; Figure 1B). The details of mature *pol pll1* embryos were examined by confocal laser scanning microscopy according to the method of Bougourd et al. (2000).

The most obvious differences between *pol pll1* and wild-type embryos were observed along the central axis of the embryo. At the apical end of the embryo, the normal dome of the shoot apical meristem (Figure 1J) was absent in *pol pll1* embryos (Figure 1O), matching our previous determination that *pol pll1* embryos lacked shoot meristem stem cells (Song et al., 2006). Along the central embryo axis below the shoot apex, *pol pll1* embryos developed files of cortex-like, relatively undifferentiated cells, instead of the radially organized cell files of cortex, endodermis, and stele (vascular cells) found in wild-type embryos (Figures 1A, 1K–1M, and 1P–1R). At the basal end of the embryo, wild-type-like sibling embryos from *pol/pol pll1*/+ parents developed root meristems, with a QC surrounded by stem cell initials (Dolan et al., 1993; Figures 1A and 1M). In contrast, *pol pll1* embryos developed no root meristem structure and lacked any evidence of root stem cell formation, in accordance with the failure of these tissues to undergo root growth postembryonically (Figure 1R). Postembryonic development at the basal tip of *pol pll1* seedlings consisted primarily of root hair differentiation at epidermal cells, as well as occasional skewed periclinal cell divisions in the epidermis at the root tip region. The loss of cells displaying starch granule staining suggests a loss of columella root cap development (Figures 1E and 1F).

In addition to a complete lack of vascular differentiation in the central embryo axis, the development of vascular strands within the cotyledons of *pol pll1* mutants was reduced compared to that of wild-type-like sibling plants (Figures 1G and 1H).

pol pll1 Mutants Are Not Auxin Insensitive

The seedling-lethal phenotype of *pol pll1* double mutant plants is in part associated with the loss of most basal embryo tissue, especially the lack of a functional root meristem and central vascular system (Figure 1B). Mutants in auxin signaling and response factors, such as *monopteros* (*mp*), *bodenlos* (*bdl*), and *auxin-resistant6* (*axr6*), exhibit superficially similar defects (Berleth and Jürgens, 1993; Hamann et al., 1999; Hobbie et al., 2000). Thus, we tested the auxin sensitivity of *pol pll1* mutants. When germinated in a growth media containing a synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D, 900 pM), *pol pll1* seedlings displayed relatively normal auxin responses by developing swollen hypocotyls and retarded expansion of cotyledons similar to wild-type seedlings, whereas *axr1* mutants were largely resistant to this treatment (Figure 1C). We next tested *pol pll1* growth over an increasing range of 2,4-D concentrations. The development of both *pol pll1* and wild-type seedlings was disrupted around 100–250 pM 2,4-D, a concentration at which *axr1-12* mutants showed little response (see Figure S1, available online). In callus-inducing media (Hamann et al., 1999), *pol pll1* and wild-type plants developed callus-like tissues within 3 weeks and failed to maintain any normal seedling structures, whereas *axr1-12* mutants still retained a basic body structure (Figure 1D).

The auxin-response mutant *mp* fails to form an embryonic root meristem, but it can form adventitious root meristems from dissected seedlings postembryonically (Berleth and Jürgens, 1993; Przemeck et al., 1996). When we subjected wild-type L*er*, *mp*, and *pol pll1* explants to root-inducing media, 47 of 47 L*er* explants and 34 of 34 *mp* explants developed adventitious roots possessing normal root meristem structures (Figures S2A, S2C, and S2E). By contrast, only 3 of 33 *pol pll1* explants developed any roots under similar conditions, and those 3 explants only formed very short roots lacking a normal root meristem structure, but containing vascular strands (Figures S2B, S2D, S2F, and S2G). Thus, the lack of a *pol pll1* embryonic root meristem could not be bypassed through growth in the appropriate hormone conditions. Taken together, these observations suggest that the defects of *pol pll1* mutants, though superficially similar to those of the auxin-insensitive mutants, are not directly related to the auxin response. These results also demonstrate a requirement for POL/PLL1 for both embryonic and postembryonic root meristem development.

POL/PLL1 Are Required for Asymmetric Divisions in Globular-Stage Embryos

Arabidopsis embryogenesis is characterized by stereotypic cell divisions that build up the basic polarities and tissues by early heart stage (Jürgens et al., 1994). To determine the earliest deviation of *pol pll1* mutants from that of wild-type, embryo sacs were cleared and the embryos analyzed. Because *pol pll1* double mutants can only be studied among the progeny of *pol/pol pll1*/+ parents, we analyzed over 700 embryos from *pol/pol pll1*/+ plants and over 800 from wild-type plants (Table S1).

Among 435 *pol/pol pll1*/+ progeny analyzed between the 2-cell and 32-cell stage of embryo development, no deviations from wild-type patterning were observed (Table S1, data not shown). At around the 64-cell stage, we observed the loss of morphological asymmetry at two sets of cell divisions among one-quarter of the *pol/pol pll1*/+ progeny (Figure 2; Table S1). At this stage, both the hypophyseal and procambial cells have just undergone asymmetric divisions, resulting in small apical and large basal cells (Jürgens et al., 1994; Figure 2; note, for the remainder of the text, we will refer to apical/basal cells oriented to the entire embryo). In one-quarter of the *pol/pol pll1*/+ progeny, both of these divisions were morphologically more symmetrical (Figure 2; Figure S3; Table S1). The progeny of these cells give rise to the tissue that develops abnormally in *pol pll1* double mutants, namely, the central vascular axis, the root stem cell organizer, and initials (Scheres et al., 1994; Figure 2; Figure S3). In later embryonic stages, the daughters of these abnormally symmetric divisions continued to show major deviations from wild-type patterning among one-quarter of the *pol/pol pll1*/+ progeny (Figure 2; Table S1).

Other defective divisions in *pol pll1* embryos include later defects in shoot apical meristem development and compromised periclinal cell divisions in the daughters of cortex/endodermis initials. Three or more daughters of the cortex/endodermis initial equivalents failed to divide periclinally in *pol pll1* embryos, suggesting a loss of this asymmetric division as well. This defect seems to be very similar to a cortex layer defect found in *scr* embryos or in the reduced root meristem subjected to CLE-containing media (Fiers et al., 2005; Wysocka-Diller et al., 2000). Whether this loss is another primary defect of *pol pll1* mutations or an indirect consequence of the early loss of proper procambial and hypophyseal cell divisions is unclear.

By following the divisions occurring within hundreds of wild-type and *pol pll1* embryos and considering the previous detailed histological analysis of embryonic root development (Scheres et al., 1994), we estimated the cell lineage pattern for the procambial and hypophyseal cell daughters in wild-type and mutant

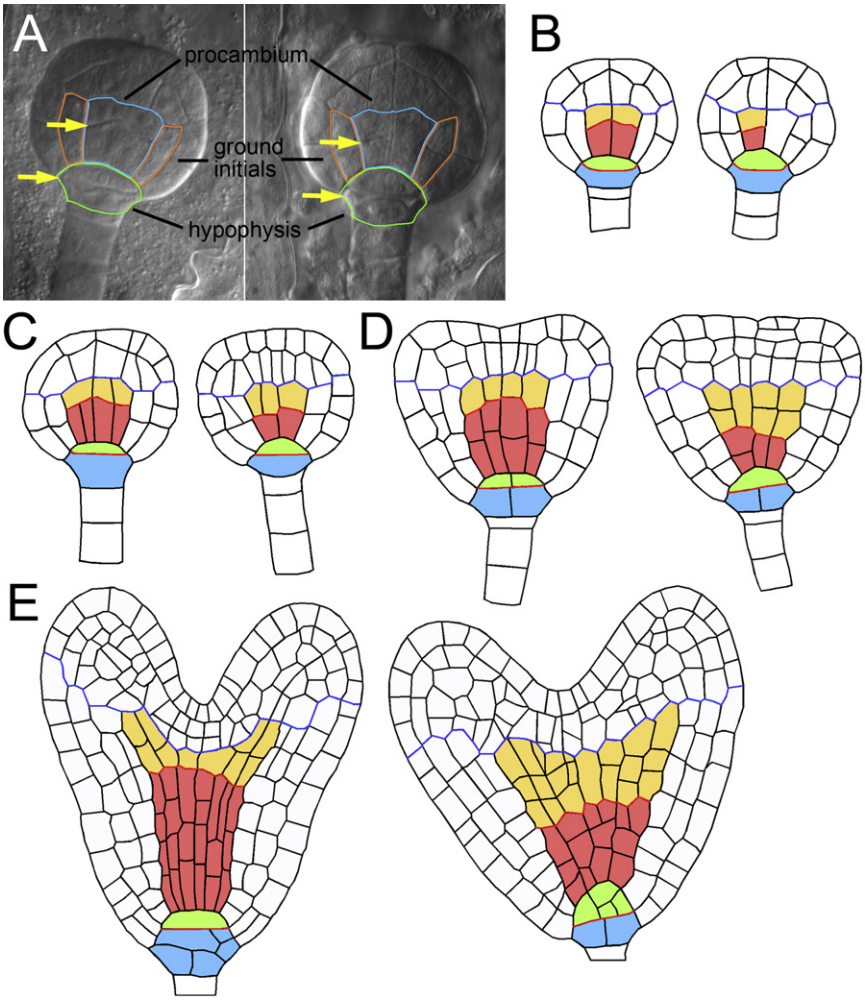

Figure 2. Procambial and Hypophyseal Cells Divide Symmetrically in *pol pll1* Embryos

(A) Control (left) and *pol pll1* (right) mid-globular-stage, cleared embryos viewed with Nomarski optics. Recently divided procambial, hypophyseal, and ground precursor cells are outlined; cell division planes for the first two are indicated by arrows.

(B–E) Cell outlines for wild-type-like (left) and *pol pll1* (right) embryos from the progeny of *pol/pol pll1*/+ plants based on Nomarski imaging (see Figure S3) at the (B) mid-globular, (C) late-globular, (D) early-heart, and (E) late-heart stage. The blue line represents the tracking of the initial apical/basal division of the apical embryonic cell (upper tier/lower tier). The red lines represent the tracking of the divisions of the procambial cell (top red line) and hypophyseal cell (bottom red line). Estimated procambial lineages are marked in orange (apical) and red (basal), whereas estimated hypophyseal lineages are marked in green (apical) and blue (basal). Lineage estimates were made based on comparisons of 1588 analyzed embryos (Table S1) and previous analyses of embryonic development (see text).

embryos (Figure 2). In wild-type embryos, the apical daughters of the procambial cell are largely quiescent and appear to give rise to a single cell layer at the upper tier/lower tier boundary. The basal daughters appear to be responsible for the bulk of the vascular development and a portion of the stem cells of the root meristem. In *pol pll1* embryos, all daughters of the procambial cell lost apparent differences in proliferation and morphology, and instead appeared to proliferate similarly, giving rise to nearly equal populations of cells from the apical and basal daughters (Figure 2). Division of the hypophyseal cell in wild-type gives rise to two very different daughters. The apical daughter forms the QC—the organizer for the root stem cell population—whereas the basal daughter gives rise to the columella stem cells and their daughters. In *pol pll1*, the daughters of the hypohyseal cells adopted neither fate (Figure 2; see below).

A lack of proper procambial and hypophyseal divisions in the *pol pll1* embryo appears to be responsible for the major defects found in the root stem cell and the vascular specification of *pol pll1* embryos and seedlings.

Fate Specification in *pol pll1* Embryos

The central axis of *pol pll1* embryos lacked the morphology of cell types normally found in this position, most noticeably vascular cells and the root stem cells. These specification defects were consistent with defects in marker gene expression in *pol pll1* mutant embryos (Figure S4). Multiple enhancer traps from the Haseloff collection that act as markers for specific root cell populations (http://www.plantsci.cam.ac.uk/Haseloff/Home.html) and reporter lines for the regulators *GL2* (Lin and Schiefelbein, 2001), *SCR* (Wysocka-Diller et al., 2000; Gallagher et al., 2004), *SHR* (Nakajima et al., 2001), and *PLT1* and *PLT2* (Galinha et al., 2007; H.J. and B. Scheres, unpublished data) were analyzed as homozygotes among the progeny of *pol/pol pll1*/+ plants (Figure 3; Figure S4). All reporters were tested for a linkage to *POL* and *PLL1* that might distort segregation.

We observed maintenance of epidermal and ground tissue specification in *pol pll1* embryos, consistent with their morphology (Figures S4A, S4B, and S4I–S4L). Interestingly, the central cells of *pol pll1* embryos were not misspecified as ground tissue despite morphological similarity. Also consistent with morphological defects, the *Q0990* enhancer trap, which is normally highly active in the central vascular axis, was inactive in *pol pll1* embryos (Figures S4O–S4R). When the expression of *J1721* in the early embryos was examined, a GFP signal could be found throughout the embryo, until the early heart stage (data not shown). After this stage, the signal from *J1721* was increasingly restricted, until it was only observed in the vascular region of wild-type embryos (Figures S4M and S4N). In *pol pll1* embryos, *J1721* expression failed to be properly restricted after the heart stage, leading to a significantly broader expression in the mutants, especially in the central axis of the embryo, perhaps associated with the failure of these cells to properly differentiate.

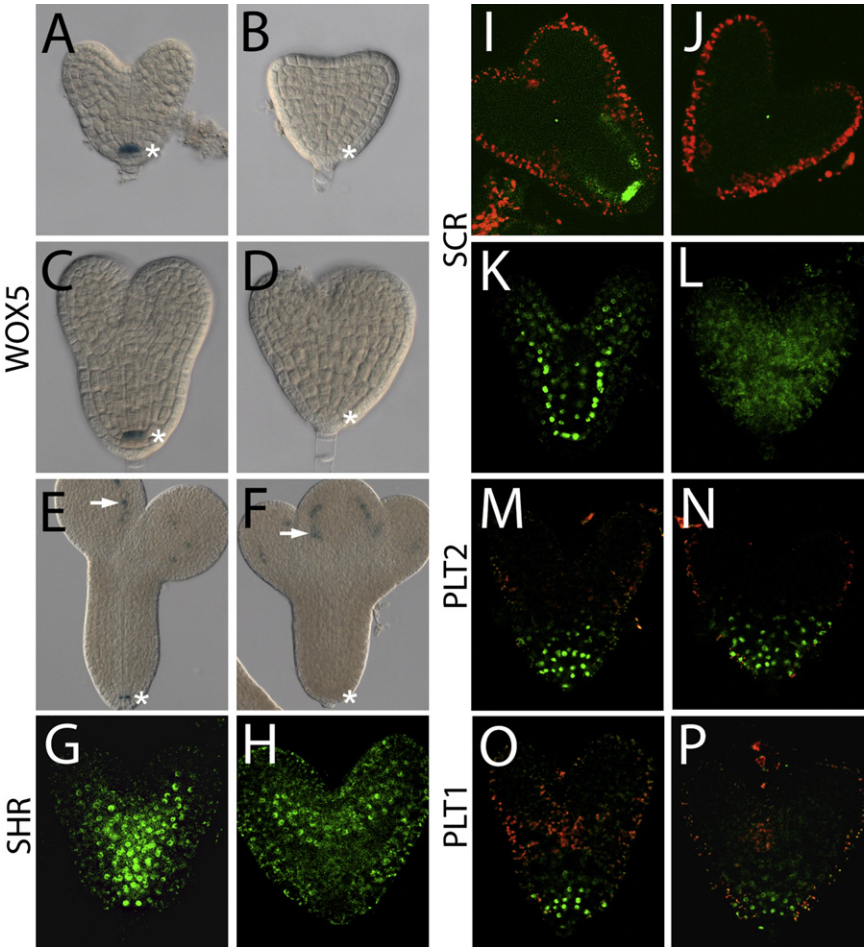

Figure 3. Embryonic Expression of *WOX5*, *SHR*, and *SCR* Is Dependent on POL/PLL1

(A–F) P_{WOX5}*:GUS* expression at the (A and B) heart stage, (C and D) late-heart stage, and (E and F) cotyledon stage in (A, C, and E) wild-type-like siblings and (B, D, and F) *pol-6 pll1-1* mutants. (G–P) Heart-stage expression in (G, I, M, and O) wild-type-like siblings, (K) *scr-4* mutants, and (H, J, L, N, and P) *pol pll1* mutants of (G and H) P_{SHR}*:SHR-GFP*, (I and J) P_{SCR}*:GFP*, (K and L) P_{SCR}*:GFP-SCR*, (M and N) P_{PLT2}*:PLT2-YFP*, and (O and P) P_{PLT1}*:PLT1-YFP*.

J2341 activity also failed to be as precisely restricted in *pol pll1* embryos compared to controls (Figures S4E–S4H).

SCR expression is necessary for the establishment of the QC at the root meristem, as well as for asymmetric divisions of ground tissue initials (Di Laurenzio et al., 1996; Sabatini et al., 2003; Wysocka-Diller et al., 2000). *pol pll1* embryos and seedlings lost detectable P_{SCR}*:GFP* and P_{SCR}*:GFP-SCR* expression, indicating a loss of both QC and endodermal cell identities, whereas P_{SCR}-driven expression was observed in the QC and the endodermis of wild-type-like siblings and rescued *scr-4* embryos (Figures 3I–3L; data not shown). The maintenance of ground tissue identity, but severe reduction of *SCR* expression, was also observed in *shr* mutants (Helariutta et al., 2000), and this finding suggests that endodermal tissue may be specified later from existing ground tissue, and that this specification is lost in both *pol pll1* and *shr* mutants.

Because *SCR* expression is under the control of SHR, we examined SHR expression as well. P_{SHR}*:SHR-GFP* expression was lost from the central axis of *pol pll1* embryos, but it was maintained in the developing vascular cells of the cotyledon primordia, whereas expression was observed in stele, endodermis, and the QC in wild-type-like siblings (Figures 3G and 3H). This result indicates that the SHR-expressing cells are not properly specified along the embryo axis in *pol pll1* embryos.

The *PLT1* and *PLT2* genes act in a pathway parallel to SCR/SHR, and the combined activity of these two pathways is required for root stem cell specification and maintenance (Aida et al., 2004). Using fully functional PLT1 and PLT2 fusions (Galinha et al., 2007; H.H. and B. Scheres, unpublished data), P_{PLT1}*:PLT1-YFP* and P_{PLT2}*:PLT2-YFP* reporters among progeny of *pol/pol pll1/+* show no significant alteration in expression in *pol pll1* double mutants (Figures 3M–3P), suggesting that PLT1/PLT2 act independently or upstream of POL/PLL1.

Evidence for a Mechanistically Conserved Shoot and Root Stem Cell Pathway

POL/PLL1 promote shoot and flower stem cells by promoting expression of the homeodomain-containing transcription factor WUS (Mayer et al., 1998; Song et al., 2006). *pol pll1* mutants fail to maintain *WUS* expression at shoot and flower meristems, whereas ectopic *WUS* expression can rescue the shoot and flower stem cell defect of *pol pll1* mutants (Song et al., 2006).

The *WUS* homolog *WOX5* is expressed in the hypophyseal cell, and, after the asymmetric division, *WOX5* expression is only retained in the apical daughter (Haecker et al., 2004). Our observation that the asymmetry of the hypophyseal division requires POL/PLL1 raises the possibility that POL/PLL1 are required for root stem cell specification because *WOX5* expression maintenance depends on the asymmetric nature of the hypophyseal division. To address this question, we crossed a previously characterized P_{WOX5}*:GUS* transgene (Sarkar et al., 2007) that recapitulates *WOX5* RNA in situ hybridization data (Haecker et al., 2004) into the *pol pll1* background. Among progeny of *pol pll1/POL PLL1* P_{WOX5}*:GUS/* P_{WOX5}*:GUS* plants (*POL* and *PLL1* are ~15 cM apart on chromosome 2), 209 embryos between the heart and bent-cotyledon stage were analyzed for both Pol$^-$ Pll1$^-$ phenotypes and GUS expression. Of 164 wild-type-like siblings analyzed, all exhibited GUS expression in the QC (Figures 3A, 3C, and 3E). Among 45 *pol pll1* embryos analyzed, none showed GUS expression at the QC (Figures 3B, 3D, and 3F). All 16 of the *pol pll1* embryos at the torpedo stage and later

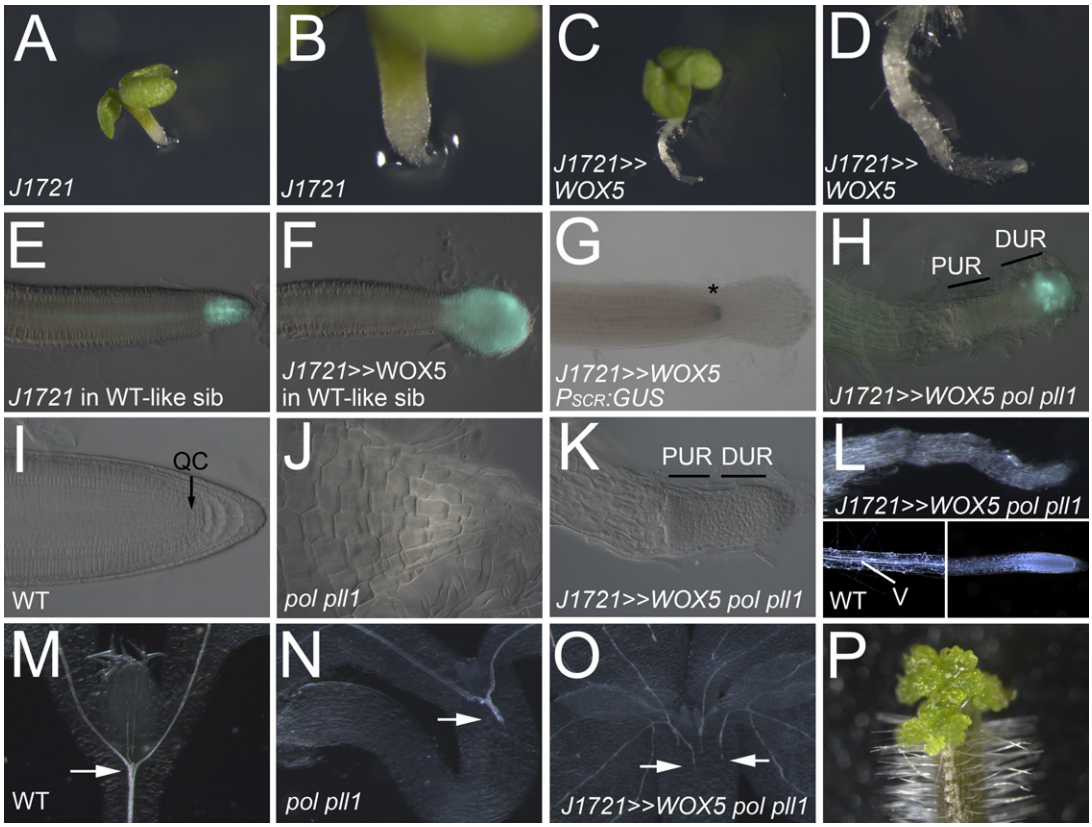

Figure 4. Ectopic *WOX5* Expression Partially Rescues the *pol pll1* Root Meristem, but Not Vascular Defects

(A and B) *pol pll1* seedlings with the *J1721* enhancer trap driver display no growth at the root.

(C and D) *pol pll1* seedlings with *J1721* > > *WOX5* expression display significant postembryonic root growth.

(E–G) (E and F) Overlays of *J1721*-driven GFP fluorescence with light microscope images of control plants with the (E) *J1721* enhancer trap alone or (F) driving *WOX5* expression. Note the accumulation of apparent distal stem cells as a result of the ectopic *WOX5* expression in (F), as evidenced by the position of P_{SCR}*:GUS* expression as a QC marker (asterisk) in (G).

(H) Light microscope image of *pol pll1* seedlings with *J1721* > > *WOX5* overlayed with *J1721*-driven GFP fluorescence. Note the development of proximal and distal undifferentiated regions (PUR and DUR).

(I–K) Higher-magnification images of (I) wild-type, (J) *pol pll1*, and (K) *pol pll1 J1721* > > *WOX5* root tips revealing the lack of normal QC organization on the rescued *pol pll1* meristems.

(L) *pol pll1 J1721* > > *WOX5* roots (top) lack vascular development compared to wild-type roots at the same magnification (bottom).

(M–O) Cleared (M) wild-type, (N) *pol pll1*, and (O) *pol pll1 J1721* > > *WOX5* seedlings reveal that the continuous vascular strands connecting the cotyledon and hypotcotyl in wild-type plants (arrow) are terminated at the base of the cotyledons in *pol pll1* and *pol pll1 J1721* > > *WOX5* plants.

(P) Control siblings with *J1721* > > *WOX5* expression accumulate apparent stem cells in aerial tissues as well.

(A) and (C), (B) and (D), (E)–(H), (I)–(K), and (M)–(O) are shown at the same magnification.

displayed GUS activity within cotyledon vascular strands, consistent with previous data showing *WOX5* expression in this tissue (Figures 3E and 3F) (Haecker et al., 2004) and demonstrating that these mutants contained the P_{WOX5}*:GUS* transgene. This indicates that *WOX5* expression in the apical hypophyseal daughter is dependent on POL/PLL1.

Although this analysis provided evidence that POL/PLL1 have a common mechanism to regulate *WUS* in shoot and flower meristems and its homolog *WOX5* in root meristem, it does not demonstrate that the lack of root stem cells in *pol pll1* embryos was the consequence of the loss of *WOX5* activity. To determine if *WOX5* could overcome the loss of root stem cells in *pol pll1* embryos, we expressed *WOX5* under control of the enhancer trap driver *J1721*, because it maintains expression at the root tip in *pol pll1* embryos, as well as in the vascular cells (Figure S4N; see below).

Among phenotypically wild-type siblings from *pol*/*pol pll1*/+ parents, *J1721* > > *WOX5* had a dramatic effect on root meristem organization (Figure 4). A large accumulation of cells occurred at the root meristem tip, leading to a bulb-shaped root (Figure 4F). Analysis of the P_{SCR}*:GUS* marker for the root stem cell organizer revealed the additional cells to be in the location of the columella root cap stem cells and daughters, whereas the rest of the root meristem was largely unaffected (Figure 4G). This suggests that *WOX5* plays a critical role in columella stem cell specification, consistent with recent findings that *WOX5* overexpression driven by the cauliflower mosaic virus *35S cis* elements represses differentiation of columella stem cells (Sarkar et al.,

2007). *J1721 > > WOX5* plants displayed massive accumulation of undifferentiated cells at shoot and flower meristems (Figure 4P).

J1721 > > WOX5 pol pll1 plants exhibited a rescue of postembryonic root growth (Figures 4C and 4D). A close examination of the growing tips of these rescued *pol pll1* mutants revealed two apparent populations of undifferentiated cells (Figures 4H and 4K). One was a distal region of cells marked by *J1721 > > GFP* expression that suggests a restoration of the distal columella stem cells. The second region of undifferentiated cells was immediately proximal to the first, but it did not show *J1721 > > GFP* expression (Figure 4H). These might correspond to the proximal stem cells found in a wild-type root; however, no QC region was observed (cf. Figures 4I, 4J, and 4K). Finally, no restoration of the central vascular strand was observed, suggesting that vascular loss in *pol pll1* is more related to the loss of asymmetric procambial division than the loss of *WOX5* expression upon hypophyseal division (Figures 4L–4O). The growth of the restored root meristem in *J1721 > > WOX5 pol pll1* plants was slow and limited, presumably as a consequence of the absence of vascular cells. In summary, *pol pll1* stem cell defects can be rescued by *WUS* at shoots and flowers or by its homolog *WOX5* in the root, suggesting a common underlying mechanism for shoot and root stem cell specification. However, rescue of *pol pll1* is incomplete, at least in terms of vascular development, suggesting multiple targets for POL/PLL1 in total during embryo development.

Attempts to rescue *pol pll1* root stem cell defects with *WUS* were complicated by embryo lethality and the conversion of basal embryo tissue to apical embryo tissue (Figure S5), a *WUS* effect previously observed (Gallois et al., 2004).

POL/PLL1 Are Required for Postembryonic Stem Cell Maintenance

Evidence for a postembryonic role for POL/PLL1 at the root meristem was also supported by *POL* complementation of *pol pll1* driven by *J1721*. This driver is active throughout the root meristem and vascular precursors during the early *pol pll1* embryogenesis (Figure S4N). Consistent with this, *J1721 > > cPOL* rescues embryo development defects of *pol pll1* mutants (Figure 5A). Postembryonically, *J1721* is restricted to the vascular cells and root cap (Figures 4E and 5E). Interestingly, *J1721 > > cPOL pol pll1* continued to develop root vascular tissue, but the root meristem consistently underwent postembryonic termination, with a loss of apparent QC specification (Figures 5B–5I). This suggests that POL is continually required at the QC for its maintenance.

The *J0571*, *J2341*, and *Q0990* drivers were unable to provide complementation of root meristem defects when used to drive *POL* expression in *pol pll1* embryos (data not shown). Thus, POL/PLL1 appear to be required within the hypophyseal cells for stem cell function.

We also expressed POL and PLL1 within the root meristem by using P_{35S}-driven overexpression. In previous studies of shoot and flower stem cells, we observed subtle flower stem cell defects from P_{35S}*:PLL1*, although we could not isolate active P_{35S}*:POL* lines (Song and Clark, 2005). When we observed root meristems of P_{35S}*:PLL1* plants, we detected no obvious changes in root meristem organization (data not shown). To amplify any subtle defect, we crossed these lines into *clv2-1*, which is thought to play a role in promoting root meristem function. Even P_{35S}*:PLL1 clv2-1* roots displayed no clear defects (Figures 5J and 5K), which suggests that our recovered P_{35S}*:PLL1* lines were selected for relatively low expression and/or that *PLL1* is primarily under posttranscriptional regulation.

Defects in Auxin Transporter Accumulation in *pol pll1* Embryos

PIN proteins are auxin-efflux carriers that are polarly localized and play crucial roles for the establishment of the basic pattern formation during embryogenesis (Galweiler et al., 1998; Okada et al., 1991). Although each single *pin* mutant displays minor embryo defects, *pin1 pin3 pin4 pin7* quadruple mutants exhibit severe defects and lack any root meristem (Blilou et al., 2005; Friml et al., 2003). PIN1 is expressed within the central embryo axis and is asymmetrically localized at the basal portion of vascular cells along the embryo axis (Steinmann et al., 1999).

The expression and localization of P_{PIN1}*:PIN1-GFP* (Benkova et al., 2003) was examined among the progeny of *pol/pol pll1*/+ plants (Figures 6A–6P). We observed normal PIN1-GFP accumulation in wild-type-like siblings (Figures 6A–6D and 6I–6L), but reduced PIN1-GFP accumulation in the central axis of *pol pll1* mutants from the late-globular-stage embryos and onward (Figures 6E–6H and 6M–6P). No obvious asymmetric localization of PIN1-GFP was observed within the central cells of *pol pll1* embryos (Figures 6M and 6N). The cotyledons of *pol pll1* exhibited PIN1-GFP expression that was equivalent to the expression in wild-type-like embryos, with apparent polar localization (Figures 6E–6H and 6M–6P). P_{PIN1}*:GUS* expression was largely unaffected in *pol pll1* embryos through the heart stage (Figure S6). After the heart stage, *pol pll1* embryos lacked the GUS signal in the central axis.

An auxin-responsive promoter containing direct repeat (DR) elements fused with GUS- or GFP-coding sequences (e.g., P_{DR5}*:GFP*) is commonly used to monitor the endogenous auxin levels in a specific region of tissue (Friml et al., 2003; Sabatini et al., 1999; Weijers et al., 2006). GUS activity from P_{DR5}*:GFP-GUS* was unaffected in *pol pll1* embryos, whereas GFP activity from P_{DR5rev}*:GFP* showed some ectopic apical activation in *pol pll1* embryos (Figures 6Q–6X). The largely unperturbed reporter gene activity suggests that either auxin transport is largely maintained despite evidence of PIN1 protein reduction (perhaps by other PINs), that these reporters are responding to other signals, or that there is auxin production at the basal tip of the embryo. This is in contrast to the auxin-insensitive mutants *mp* and *bdl*, which lack a *DR5*-driven signal at the basal embryo tip (Sabatini et al., 1999; Ulmasov et al., 1997).

POL Expression during Embryogenesis

To determine whether the expression pattern of the *POL* during embryogenesis correlates with the embryo defects of *pol pll1* mutants, P_{POL}*:GUS* reporter gene expression was examined in embryos. We have previously shown that this reporter matches RNA in situ hybridization results within the shoot meristem region and have rescued *pol pll1* mutant phenotypes by driving *POL* and *PLL1* under control of these *cis* elements (Song and Clark, 2005; data not shown). No significant P_{POL}*:GUS* expression was observed until the protoderm stage. P_{POL}*:GUS* expression was first detected from the midglobular stage, especially in the

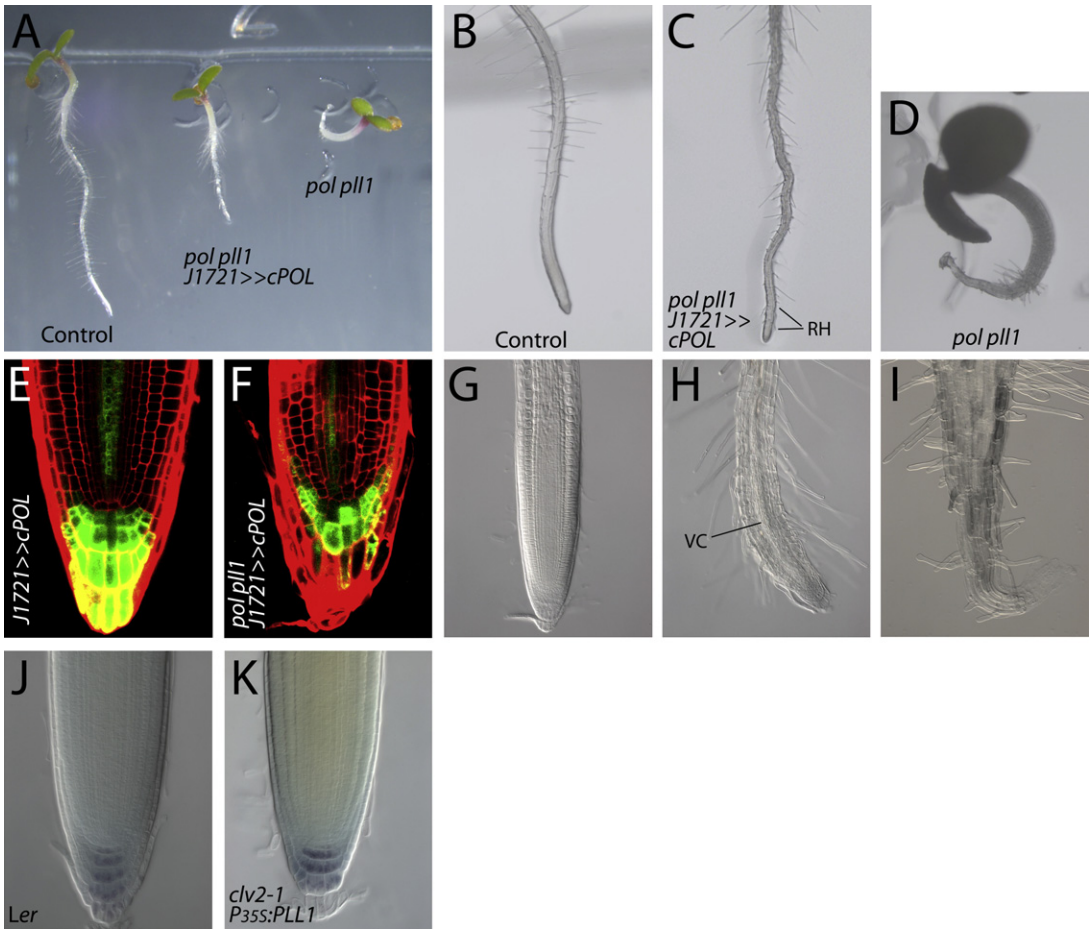

Figure 5. The Rescue of *pol pll1* Root Meristem Defects Depends on Continuous *POL* Expression at the Root Meristem

(A) A wild-type-like sibling control (left), a *J1721 > > cPOL pol pll1* seedling (center), and a *pol pll1* seedling (right) shown at 3 dag.

(B–D) A (B) wild-type-like sibling, a (C) *J1721 > > cPOL pol pll1* seedling, and a (D) *pol pll1* seedling at 5 dag. Note the development of root hairs (RH) at the root tip in the *J1721 > > cPOL pol pll1* seedling.

(E and F) Confocal images of *J1721* in a (E) wild-type-like sibling and (F) *1721 > > cPOL pol pll1* at 4 dag.

(G–I) Higher-magnification views of root tips for a (G) wild-type-like sibling, a (H) *J1721 > > cPOL pol pll1* seedling, and a (I) *pol pll1* seedling at 7 dag. Note the vascular cell (VC) development close to the root tip in the *J1721 > > cPOL pol pll1* seedling.

(J and K) A 7-day-old wild-type (J) L*er* and (K) *clv2-1* P_{35S}*:PLL1* with lugol staining.

basal region. The initiation and localization of P_{POL}:*GUS* expression correlated well with the earliest apparent defects of *pol pll1* mutants. P_{POL}:*GUS* expression within later-stage embryos was highest along the central embryo axis, especially the shoot and root meristems (Figure S7), consistent with a dual requirement for stem cell specification at both locations.

DISCUSSION

POL/PLL1 Are Required for Key Embryonic Asymmetric Divisions

The earliest phenotypes and the apparent primary defect in *pol pll1* embryos are the loss of morphological asymmetry in two sets of cell divisions in the basal portion of the early embryo. The procambial and hypophyseal cells normally undergo divisions that are highly asymmetric in terms of both morphology and developmental fate. For the procambial cells, the apical daughter is both smaller and largely quiescent, whereas the basal daughter is highly proliferative and gives rise to both the vascular stem cells at the root meristem and the earliest set of vascular tissues in the central embryo (apical and basal designations in relation to the whole embryo). The apical daughter of the hypophyseal cell forms the eventual QC that acts as the organizer for the root meristem stem cells, whereas the larger basal daughter gives rise to the columella root cap and its initials. Together, these two sets of asymmetric divisions form the root meristem stem cells as well as the organizing QC.

In *pol pll1* embryos, these divisions lose morphological asymmetry and evidence of proper specification. Interestingly, the resulting divisions in *pol pll1* give rise to cells that adopt neither the apical fate nor the basal fate normally associated with these cells, based on a variety of cell specification markers tested. Thus, POL/PLL1 are critical for the proper division and specification of the procambial and hypophyseal cell, and they perhaps act by establishing their cell asymmetry, or by controlling the specification of the parent cell prior to division.

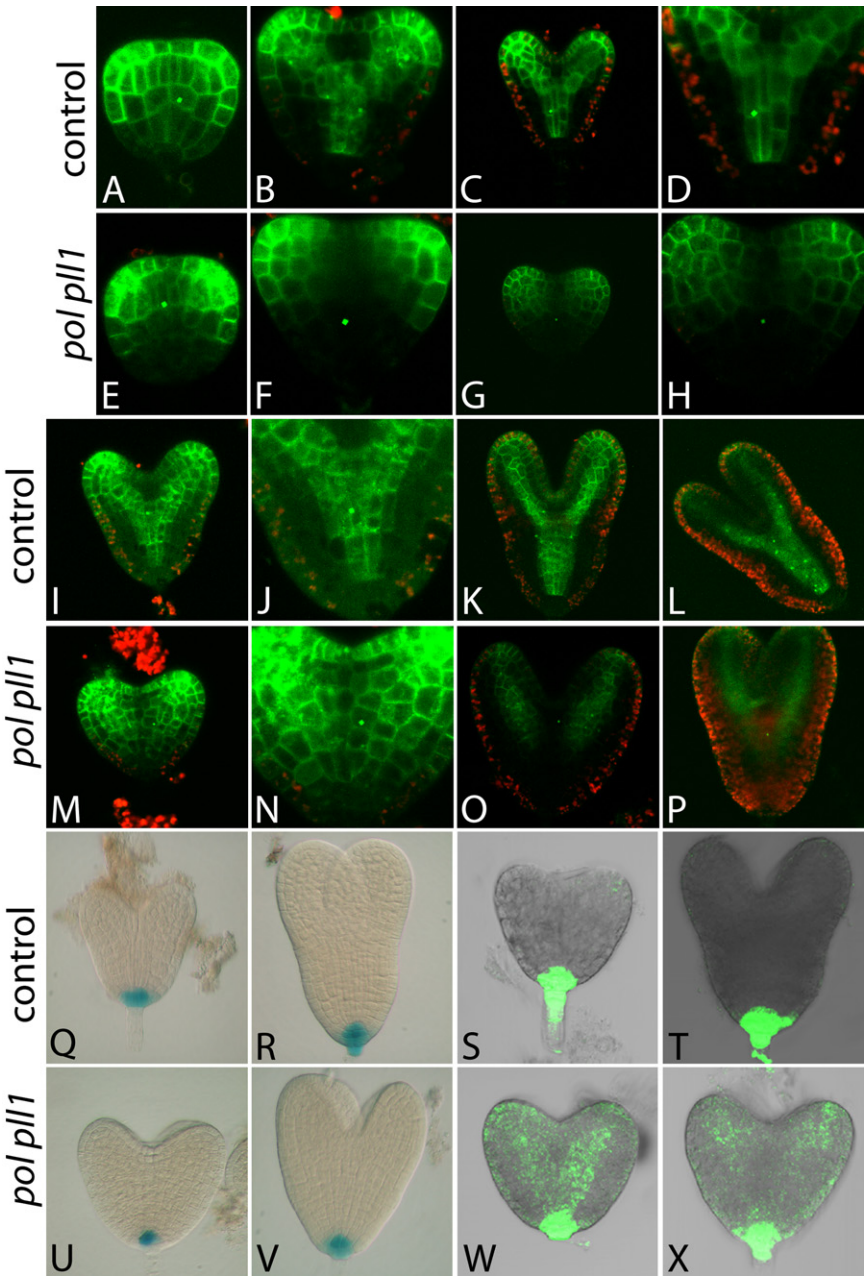

Figure 6. Loss of PIN1-GFP Accumulation in the Central Axis of *pol pll1* Embryos

(A–D and I–L) Wild-type-like embryos and (E–H and M–P) *pol pll1* embryos among the progeny of P_{PIN1}*:PIN1-GFP pol pll1*/+ plants observed with confocal microscopy. Embryos were observed at the (A and E) late-globular, (B and F) early-heart, (C, D, G–J, M, and N) mid-heart, (K and O) late-heart, and (L and P) torpedo stages. The signal intensity in (I), (J), (M), and (N) were amplified to visualize a weak GFP signal in the central axis. A GUS signal from P_{DR5}*:GFP-GUS* at the (Q and U) heart and (R and V) torpedo stages in (U and V) *pol pll1* embryos and (Q and R) wild-type-like siblings. An overlay of Nomarski images and CLSM images of a GFP signal from P_{DR5rev}*:GFP* at the (S and W) heart and (T and X) late-heart stages in (W and X) *pol pll1* embryos and (S and T) wild-type-like siblings. (A), (B), (D)–(F), (H), (J), and (N); (C), (G), (I), (K), (M), and (O); (L) and (P); (Q), (R), (U), and (V); and (S), (T), (W), and (X) are shown at the same magnification, respectively.

POL and PLL1 Function Separately in Vascular and Stem Cell Specification

Because *pol pll1* affects divisions and specification of two different cells, one must consider that a defect in one cell (e.g., the hypohyseal cell) may be an indirect consequence of a defect in the other (e.g., the procambial cell). Several lines of evidence suggest that this is not the case, and that POL/PLL1 act separately in each cell. First, *J1721 > > cPOL* rescue of *pol pll1* revealed that vascular *POL* expression and vascular development are not sufficient to maintain root meristem function. Second, rare adventitious roots from *pol pll1* explants developed vascular cells, but failed to establish a functional meristem. Third, *J1721 > > WOX5* rescued the root meristem defect of *pol pll1* mutants, but had no effect on the lack of vascular specification. Fourth, the *POL* promoter was activated similarly in both cells. Fifth, the hypohyseal cell division precedes that of the procambial cells.

POL/PLL1 Reveal Similarity in Root and Shoot Stem Cell Specification

Whereas existing mutations altering stem cell specification in *Arabidopsis* affect either the shoot/flower or the root meristem, but not both, *pol pll1* double mutations lead to a loss of all stem cell populations in *Arabidopsis*. POL/PLL1 represent a pathway shared at both the shoot and root for stem cell maintenance.

At both the root and shoot/flower stem cell populations, POL/PLL1 are required for the expression of *WUS* or its close homolog *WOX5*. In nascent shoot or flower meristems, *WUS* expression is immediately lost in *pol pll1* backgrounds, and this loss is the primary reason for the loss of shoot/flower stem cells in *pol pll1* mutants (Song et al., 2006). In the embryonic root meristem, POL/PLL1 are required for the expression of *WOX5*. *WOX5* is required for stem cell specification distal to the QC, and it may be required for proximal stem cells as well (Sarkar et al., 2007). The lack of root growth and root stem cells in *pol pll1* can be bypassed by the expression of *WOX5* at the root tip, indicating that the loss of *WOX5* is the primary cause of the stem cell defects (but not vascular defects) in these mutants.

The commonality of a POL/WOX pathway in root and shoot/flower stem cells may suggest a conserved evolutionary origin for the root meristem, which evolved later than the shoot meristem (Friedman et al., 2004). It is also possible that the origin of the root meristem was separate, but that the POL/WOX pathway was co-opted for the newly evolved stem cells at the root.

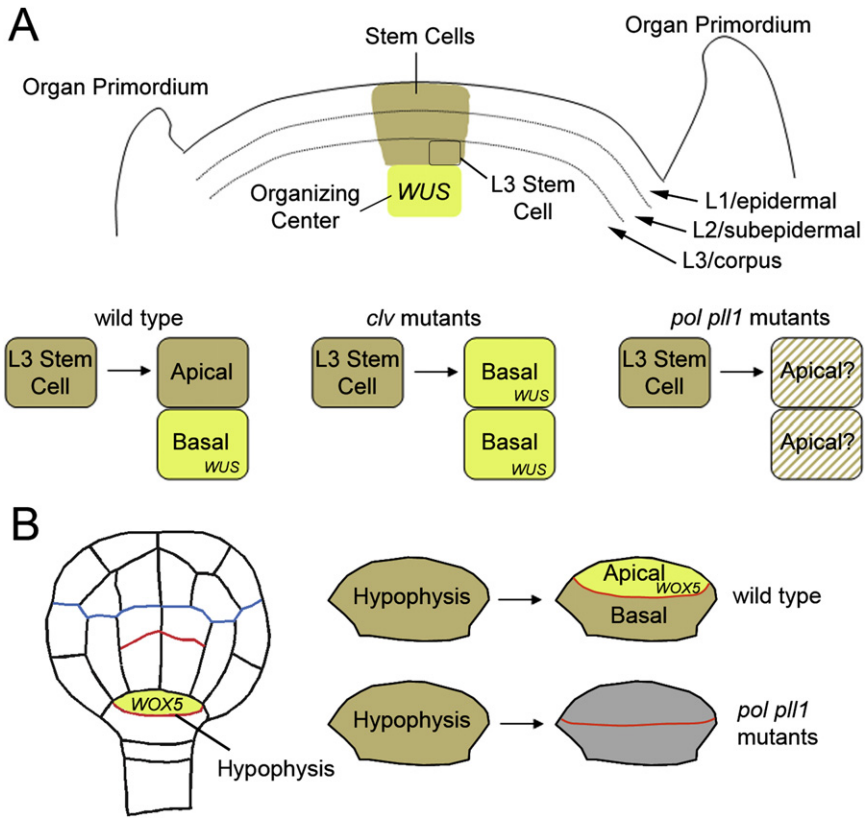

Figure 7. Model for a Conserved Mechanism for Shoot and Root Stem Cell Specification

(A) Control of shoot (and flower) stem cells originates from the organizing center (OC), which is specified by *WUS* expression. The shoot OC is derived from asymmetric divisions of L3 stem cells. The asymmetry of *WUS* expression is lost in *clv* mutants, with both daughters exhibiting *WUS* expression, whereas *pol pll1* mutants fail to maintain *WUS* expression in either daughter.

(B) The quiescent center of the root meristem plays an analogous role in organizing root stem cells and is derived from asymmetric division of the hypophyseal cell and linked to *WOX5* expression. In *pol pll1* mutants, the hypophyseal cell divides symmetrically, and both daughters lack *WOX5* expression and evidence of apical/basal fate specification.

It is clear that whereas the POL/WOX pathway operates similarly at the shoot and root meristems, other pathways remain specific for one or the other meristem based on current evidence. At the shoot meristem, regulators such as SHOOTMERISTEMLESS and ULTRAPETALA represent separate pathways from POL/WOX, and they play no apparent role in root stem cell specification (Barton and Poethig, 1993; Carles et al., 2004; Clark et al., 1996). Similarly, in the root, the roles of factors such as SCR, SHR, and PLT1/PLT2 are critical, but limited, as far as is known, to root stem cells (Aida et al., 2004; Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003).

A Model for Asymmetric Divisions Required for Stem Cell Maintenance

We propose a model based on the observations that POL/PLL1 are required for at least some aspects of the asymmetric division of the hypophyseal cell, and that they are required for shoot and root stem cell specification through regulation of *WUS* and *WOX5*.

At the shoot meristem, stem cell specification and *WUS* expression are tightly linked to asymmetric divisions of L3 stem cells (Figure 7A). These cells undergo apical/basal divisions in which the apical daughter remains a stem cell, whereas the basal daughter switches toward differentiation and expresses *WUS*, forming the OC (Laux, 2003; Mayer et al., 1998). In *clv* mutants, this asymmetry is lost, in that both the apical and basal daughters express *WUS* (Schoof et al., 2000). Whether these cells lose other aspects of asymmetry is unknown. In *pol pll1* mutants, *WUS* expression is lost immediately after meristem initiation (Song et al., 2006). Thus, CLV signaling acts through POL/PLL1 to control at least some aspects of the asymmetric character of this key stem cell division.

Similarly, in the root, the organizing QC for the stem cells is established and maintained through asymmetric divisions. During embryo development, the hypophyseal cell undergoes an asymmetric division to establish the QC and the distal columella root cap. This asymmetric division is linked to the asymmetric expression of *WOX5*, which is only maintained in the apical daughter and requires POL/PLL1 (Figure 7B).

We propose that POL/PLL1 act downstream of a receptor signaling system to establish the asymmetric character of these stem cell divisions. In the shoot meristem, CLV signaling could provide the information necessary to orient the L3 cell prior to division because the putative ligand CLV3 is expressed to the apical side of the L3 cell. In the hypophyseal cell division to establish the root meristem, we propose that another receptor signaling system acts through POL/PLL1 to provide asymmetry to the division. Pol^{-} Pll1^{-} phenotypes would suggest that these factors are required for all root stem cells, including the maintenance of postembryonic root meristems.

EXPERIMENTAL PROCEDURES

Plant Materials

The *pol-6*, *pll1-1*, and *pol-6 pll1-1* mutants were previously described (Song and Clark, 2005; Song et al., 2006; Yu et al., 2003). Auxin-response mutants *monopteros* (*mp*)^{U55}, *auxin-resistant* (*axr*) *1-3*, and *axr1-12*; enhancer trap lines *J0571*, *J1092*, *J1721*, *J2341*, and *Q0990* (donated by J. Haseloff); and P_{SCR}*:GFP* and P_{SCR}*:GFP-SCR scr-4* lines (donated by P. Benfey) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). The P_{GL2}*:GFP* line (Lin and Schiefelbein, 2001) was provided by J. Schiefelbein. P_{SHR}*:SHR-GFP* (Nakajima et al., 2001) and P_{SCR}*:GUS* (Di Laurenzio et al., 1996) were provided by P. Benfey. P_{PIN1}*::PIN1:GFP*, P_{PIN1}*:GUS* (Benkova et al., 2003), and P_{DR5}*:revGFP* (Friml et al., 2003) were obtained from the Nottingham *Arabidopsis* Stock Center (NASC). P_{WOX5}*:GUS* (Sarkar et al., 2007) was provided by T. Laux. The P_{DR5}*:GFP-GUS* line was provided by M. Prigge. *Arabidopsis* lines containing the reporter genes were crossed to *pol-6 pll1-1*/+,

and, for each, an F3 seed pool that was homozygous for the reporter gene and *pol-6*, and heterozygous for *pll1-1*, was selected and observed. Plants were grown as described previously (Song and Clark, 2005).

The screening for homozygous enhancer trap lines and P_{SCR}:*GFP* lines utilized the primers GFPf/GFPr for polymerase chain reaction (PCR) genotyping (see Table S2 for all oligonucleotide primer sequences). The screening for homozygous lines for P_{SCR}:*GFP-SCR* and P_{SHR}:*SHR-GFP* utilized the primers SCRseq1r/GFPCtemif and SHRseq1f/GFPNtermir for PCR genotyping, respectively.

Analysis of the Auxin Response

Seeds of *pol-6 pll1-1*, *axr1-3*, *axr1-12*, Col, and L*er* were surface sterilized and germinated on half-strength MS media (Sigma) prepared as described (Song et al., 2006) containing various concentrations of 2,4-D or containing 1 μg/ml 2,4-D and 0.25 μg/ml kinetin, inducing callus formation (Hamann et al., 1999). To test auxin-induced adventitious root formation, seedlings of *pol-6 pll1-1*, *mp*, and L*er* were dissected and cultured on half-strength MS media containing 3 μg/ml indole butyric acid (IBA) as described (Berleth and Jürgens, 1993).

Phenotypic Analysis

For the observation and quantitative analysis of the embryos of *pol pll1*, embryo sacs collected from siliques of *pol-6 pll1-1*/+ plants were cleared in a solution containing an 8:3:1 mixture of chloral hydrate:water:glycerol and were visualized with a Zeiss Axioskop microscope (Mayer et al., 1991). Root tips of *pol-6 pll1-1* and wild-type 3-day-old seedlings were stained with Lugol solution (Sigma) and were cleared with chloral hydrate as described (Willemsen et al., 1998). Photographs were taken by using a Nikon Coolpix 995 digital camera on a Zeiss Stemi SV11 stereoscopic microscope, a Nikon Optiphot-2 compound microscope, or a Zeiss Axioskop microscope equipped with differential interference contrast optics. A Zeiss Imager D1 equipped with an Axiocam HRC CCD camera and a Leica MZ16F equipped with a DFC420 CCD camera were used for microscopy. Confocal microscopy for mature embryos stained with aniline blue was performed as described (Bougourd et al., 2000). For the visualization of GFP, a Zeiss LSM510 or LSM510 meta was used as described previously (Lee and Schiefelbein, 1999). β-glucuronidase activity was examined as described (Sessions et al., 1999). Embryos at various stages were dissected from embryo sacs with tweezers and incubated in the GUS staining solution.

Ectopic Expression of *WUS*, *WOX5*, and *POL* in *pol-6 pll1-1* Mutants

The cDNA fragments for *WUS* and *WOX5* were PCR amplified by using the primer pairs WUSBamHIN/WUSSpeIC and WOX5BamHIN/WOX5SpeIC, respectively. The cDNA fragments were cloned into pGEM-Teasy vector (Promega), and their sequences were confirmed. The cDNA fragments for *WUS* and *WOX5* digested with BamHI/SpeI and a *POL* cDNA fragment (Song and Clark, 2005) digested with BamHI were inserted into pCB302 containing the 6× UAS promoter (provided by S.-H. Kwak) and the *nos* terminator linearized with the same restriction enzymes. *pol-6* plants were transformed with these constructs as described (Clough and Bent, 1998). Several independent transgenic plants were crossed to *J1092* and *J1721* enhancer trap lines also heterozygous for *pol-6* and *pll1-1*. F2 progenies were analyzed for their phenotypes.

SUPPLEMENTAL DATA

Supplemental Data include detailed analysis of auxin responses in pol pll1 embryos and control plants, adventitious rooting, cleared embryos, marker gene expression, WUS overexpression, PPIN1:GUS activity and PPOL:GUS activity and are available at http://www.developmentalcell.com/cgi/content/full/15/1/98/DC1/.

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